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Proteoglycan synthesis in bovine articular cartilage explants exposed to different low-frequency low-energy pulsed electromagnetic fields[1]

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Summary

Objective: To investigate the role of pulsed electromagnetic field (PEMF) exposure parameters (exposure length, magnetic field peak amplitude, pulse frequency) in the regulation of proteoglycan (PG) synthesis of bovine articular cartilage explants.

Methods: Bovine articular cartilage explants were exposed to a PEMF (75 Hz; 2 mT) for different time periods: 1, 4, 9, 24 h. Then, cartilage explants were exposed for 24 h to PEMFs of different magnetic field peak amplitudes (0.5, 1, 1.5, 2 mT) and different frequencies (2, 37, 75, 110 Hz). PG synthesis of control and exposed explants was determined by Na_2-$^{35}SO_4$ incorporation.

Results: PEMF exposure significantly increased PG synthesis ranging from 12% at 4 h to 17% at 24 h of exposure. At all the magnetic field peak amplitude values, a significant PG synthesis increase was measured in PEMF-exposed explants compared to controls, with maximal effect at 1.5 mT. No effect of pulse frequency was observed on PG synthesis stimulation.

Conclusions: The results of this study show the range of exposure length, PEMF amplitude, pulse frequency which can stimulate cartilage PG synthesis, and suggest optimal exposure parameters which may be useful for cartilage repair in *in vivo* experiments and clinical application.

Key words: Electromagnetic field, Proteoglycans, Articular cartilage, Dosimetry.

Introduction

Low-frequency low-energy pulsed electromagnetic fields (PEMFs) display several effects on a variety of biological tissues, including bone and cartilage. To date, much work has investigated and confirmed the activity of PEMFs on bone tissue. Several *in vitro* and *in vivo* studies have shown that PEMFs can modify some relevant physiological parameters of bone cells, such as proliferation[1], differentiation[2], the synthesis of extracellular matrix (ECM) components[2–4] and the production of growth factors[2] and can stimulate osteogenesis in animals[5,6]. Finally, PEMFs are widely used to promote the bone healing of ununited fractures in humans[7–9].

Further, clinical observations suggest the possibility that PEMF exposure might be useful for the treatment of degenerative cartilage disorders such as osteoarthritis (OA)[10–13].

Several studies have investigated the effects of PEMFs on cartilage cells and tissue showing that PEMFs can stimulate chondrocyte proliferation[14–16] and increase the amount of cartilage ECM components[17–20]. It has been observed that the stimulation of chondrocyte proliferation, induced by PEMFs, is dependent on culture conditions such as cell density and the amount of serum in the medium[16]. Moreover, PEMFs stimulate proteoglycans (PGs) synthesis *in vivo* and in bovine adult cartilage explants cultured *in vitro*[18,21]; nevertheless this effect has not been observed in chondrocyte monolayers[20,22].

PGs are fundamental components of cartilage ECM and PG loss from the tissue is observed in OA[23]. In our previous studies, PG synthesis has been investigated in cartilage explants as they seem to be an appropriate *in vitro* model to investigate the effects of PEMFs on cartilage ECM metabolism. We have shown that PEMFs can stimulate PG synthesis, without affecting their degradation, in bovine full-thickness cartilage explants cultured *in vitro*[19,20], suggesting that the PEMF-induced stimulation of PG synthesis might be useful to preserve cartilage integrity and function.

Furthermore, it has been shown that PEMF exposure can reduce OA progression in animal models[24,25]. These effects have been interpreted by considering that PEMF exposure increases adenosine binding to A2a adenosine receptors in

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human inflammatory cells, thus resulting in an adenosine-agonist effect which might explain the chondroprotective effect exerted by PEMFs[25–27]. The studies reported above indicate that the effects of PEMFs are in part dependent on biological experimental conditions such as cell density and fetal calf serum concentration. Indeed, it is known that also biophysical exposure conditions including magnetic field peak amplitude and frequency, as well as exposure length may influence the biological response of cells and tissues to PEMF stimulation[1,21,26,28–30].

To our knowledge, few investigators have addressed the relative importance of PEMF physical parameters on cartilage response by dose–response curves. This study was designed to identify the exposure conditions that could elicit the highest PG synthesis, in bovine cartilage explants; exposure length, magnetic field amplitude and pulse frequency were investigated.

Method

CARTILAGE EXPLANT CULTURES

Explants of bovine articular cartilage were aseptically dissected from the metacarpophalangeal joints of 14–18-month-old animals (Limousine breed), as previously described[19,20]. Full-thickness cartilage discs were obtained from the articular surface using a 4-mm dermal punch (Stiefel Laboratories, Milan, Italy). Control (unexposed) and test (exposed) explants were paired at harvest and originated from adjacent sites on the joint surface. Four groups of samples (each group from each site on the joint surface) were harvested from each animal donor. In each group, the explants were randomly subdivided into control (unexposed) and test (exposed) explants. Five different animals were used for each set of experiments. Explants (three/well) were cultured in 0.5 ml culture medium in multiwells (Nunc, Denmark, 6.6×6.6 cm, 1.6 cm the diameter of each well). Before PEMF exposure, all explants were allowed to equilibrate in culture for 48 h in DMEM/F12 supplemented with 10% FBS and antibiotics (penicillin 100 units/ml, streptomycin 0.1 mg/ml) (Life Technologies Paisley, UK) (complete medium) and for an additional 48 h in medium without serum, at 37°C in an atmosphere of 5% CO_2.

PEMF APPARATUS

The apparatus for PEMF exposure was the same as that used in previous studies[1,15,19,20,26]. It consisted of a pair of circular Helmoltz coils of copper wire placed opposite to each other and in a signal generator (Igea, Carpi, Italy). The multiwell plates were placed between this pair of Helmoltz coils, so that the plane of the coils was perpendicular to the multiwell plates, and the direction of the induced electric field was perpendicular to the direction of the magnetic field.

The power generator produced a pulsed signal with the following parameters: the pulse duration was 1.3 ms and the frequency was adjustable from 2 to 110 Hz. The amplitude of magnetic field, varying from 0.5 to 2 mT, and the corresponding peak induced voltage value, were detected, between the two coils placed at different distances from each other, by the Hall probe of the Gaussmeter (LE, Gaussmeter DG500, USA), and by a standard coil probe (50 turns, 0.5 cm internal diameter of the coil probe, 0.2 mm ∅ copper wire) of a Tektronix 720A oscilloscope (Tektronix, Inc., Beaverton, OR), respectively (Fig. 1). The shape of the induced electric field and its impulse length

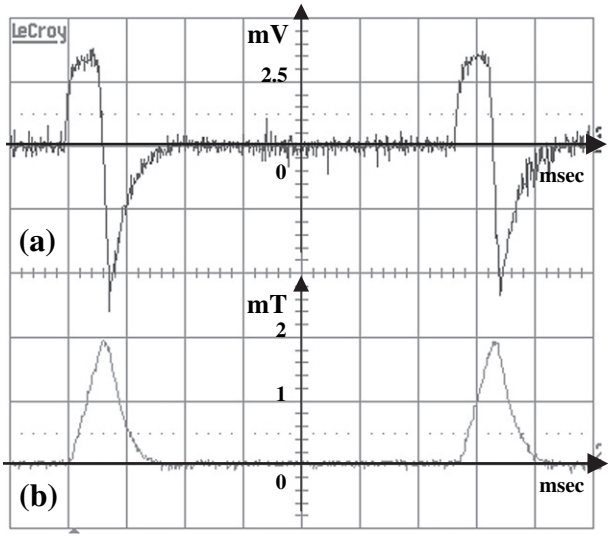

Fig. 1. (a) Waveform of the electric field induced in the standard coil described in Methods. The shape of the electric field equals the shape of the signal imposed on tissue. The amplitude of the electric field measured is a function of the rate of change in the magnetic field: d*B*/d*t*. (b) Rate of change of the magnetic field. In abscissa each division is 2 ms.

were kept constant. Inside this area, the magnetic field was uniform and it was focused on the multiwell plate, which was placed between the two coils.

PEMF EXPOSURE CONDITIONS

In the first set of experiments PG synthesis was investigated vs exposure length. To this aim, at the end of the equilibration period in culture (time 0), the explants were exposed for 1, 4, 9, and 24 h to a PEMF with the same characteristics as used in previous studies (75 Hz; 2 mT)[19,20]. PEMF exposure was performed in the presence of 10% FBS in the culture medium (0.5 ml/well) and the explants were maintained in culture for 24 h, independently of the exposure time. The medium was changed at the beginning of the exposure both in unexposed and exposed explants.

Based on the results of the first set of experiments, the most effective exposure time was chosen and PG synthesis was measured in cartilage explants exposed to PEMFs of different magnetic field peak values: 0.5, 1, 1.5, 2 mT. Finally, the most effective exposure time and peak field values were selected and PG synthesis was measured with PEMFs of different frequencies (2, 37, 75, 110 Hz). All experiments were performed under the same standard culture conditions described above. PEMF-unexposed explants (controls) were placed in the same incubator as the exposed explants at a distance where no difference from background magnetic field was observed, when the PEMF generator was turned on. All treatments were performed with triplicate wells.

PG SYNTHESIS

PG synthesis was measured as radioactive sulfate incorporation into glycosominoglycans (GAGs), which are biochemical components of PGs[20,21]. Whatever the field characteristics and the exposure time, PG synthesis was evaluated during the 24 h of culture from the beginning of

the exposure (time 0). 5 μCi/ml of Na_2-$^{35}SO_4$ (2.2 mCi/ml) (Amersham Pharmacia Biotech, Buckinghamshire, England) was added to the culture media of both unexposed and exposed explants at time 0. After the radiolabeling, explants were rinsed and digested in 20 mM phosphate buffer (pH 6.8) containing 4 mg/ml papain (Sigma–Aldrich S.r.l., Milan, Italy) at 60°C for 12 h[31]. The content of ^{35}S-labeled newly synthesized PGs (^{35}S-PGs) was measured following precipitation of the ^{35}S-PGs with cetylpyridinium chloride (Sigma–Aldrich S.r.l., Milan, Italy) and filtration onto glass fiber filters (Whatman GF/C)[32]. Filters were dried and radioactivity was quantitated by liquid scintillation counting.

TOTAL DEOXYRIBONUCLEIC ACID DETERMINATION

Aliquots from cartilage explants papain-digests were analyzed for DNA content by the fluorometric method of Labarca and Paigen[33].

STATISTICAL ANALYSIS

Data were expressed as the mean values ± S.E. Comparisons between groups were performed using Student's *t* test, with $P \leq 0.05$ considered significant.

Results

EXPOSURE LENGTH EFFECTS ON PG SYNTHESIS

Similarly to what previously reported[20], no changes were observed in DNA content between control and exposed explants in all conditions tested; PG synthesis is reported as cpm/μg DNA.

When cartilage explants were exposed to 2 mT, 75 Hz PEMF, PG synthesis increase over controls ranged from 12% at 4 h to 17% at 24 h of exposure (Fig. 2). One-hour exposure did not significantly increase PG synthesis.

MAGNETIC FIELD PEAK INTENSITY AND FREQUENCY EFFECTS ON PG SYNTHESIS

On the basis of the results obtained in the first set of experiments, explants were exposed for 24 h to PEMF with a magnetic field peak amplitude ranging from 0.5 to 2 mT

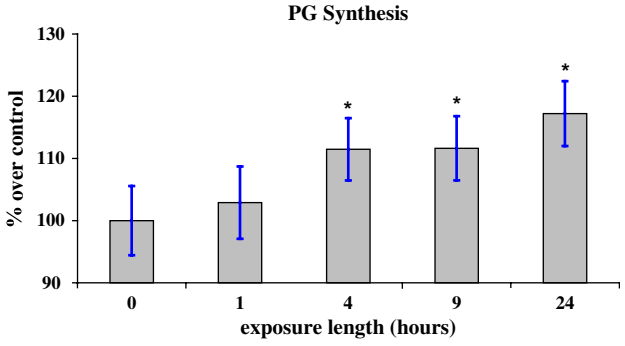

Fig. 2. Exposure time-dependent effects on PG synthesis. Cartilage explants were exposed for different exposure times (1, 4, 9, 24 h) to a selected field (75 Hz, 2 mT). ^{35}S-PGs incorporation values were normalized to corresponding control values (100%). Differences were considered significant at $P \leq 0.05$. * Indicates statistical significance vs control.

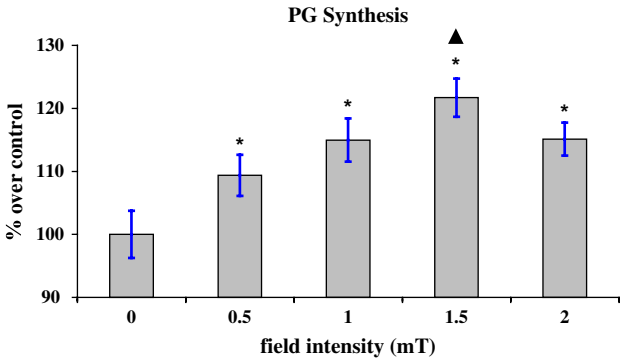

Fig. 3. Magnetic field peak amplitude-dependent effects on PG synthesis. Cartilage explants were exposed for 24 h to different fields (0.5, 1, 1.5, 2 mT, 75 Hz). ^{35}S-PGs incorporation values were normalized to corresponding control values (100%). Differences were considered significant at $P \leq 0.05$. * Indicates statistical significance vs control. ▲ indicates statistical significance vs 0.5 mT.

(Fig. 3). At all the magnetic field peak amplitudes, a significant PG synthesis increase was measured in PEMF-exposed explants compared to controls. Maximum increase (21% over controls) was observed when peak field amplitude was set at 1.5 mT and it was significantly higher than that observed at 0.5 mT (9% over controls). At 2 mT the increase in PG synthesis (15% over controls) was slightly lower than at 1.5 mT, although this difference was not significant.

Then, the relationship between PEMF frequency and PG synthesis was investigated at the magnetic field peak intensity of 1.5 mT and the exposure length of 24 h (Fig. 4). At all the frequencies investigated a significant increase in PG synthesis over controls was observed, however no significant difference in PG synthesis levels was observed among different frequencies tested.

Discussion

The strict control of both culture conditions and exposure physical characteristics are of critical importance when investigating the effect of PEMFs. In fact, several studies show that the PEMF-induced proliferative and differentiative

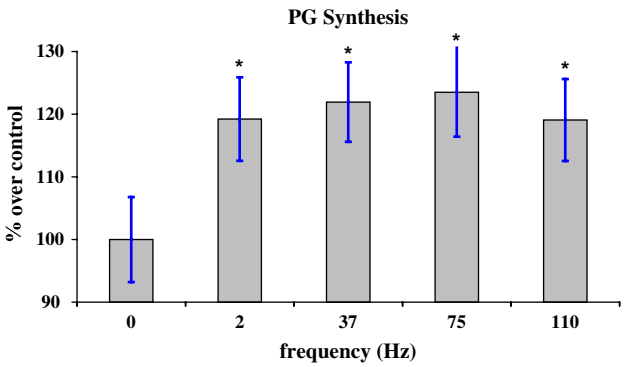

Fig. 4. Frequency-dependent effects on PG synthesis. Cartilage explants were exposed for 24 h to different fields (2, 37, 75, 110 Hz, 1.5 mT). ^{35}S-PGs incorporation values were normalized to corresponding control values (100%). Differences were considered significant at $P \leq 0.05$. * Indicates statistical significance vs control.

effects are related to the cell type[1,15], the differentiative stage[34], and the culture conditions[1,16,35]. Furthermore, PEMF biological effects are dependent on exposure time and on physical parameters characterizing the electromagnetic field[1,21,26,28–30,36]. *In vitro* and *in vivo* studies show that PEMFs can modify cartilage metabolism[14–20] and ultimately prevent OA progression in guinea pigs[24,25]. Thus, it is of paramount importance to identify the most effective exposure conditions at eliciting PG synthesis in cartilage explants.

Our previous experiences[19,20] and clinical investigations[8] have been conducted with the electric field signal whose characteristics are described in Fig. 1(a), in particular the amplitude of the electric field is kept constant for the whole length of the impulse width, 1.3 ms. In this study, keeping the pulse waveform constant, dose–response curves were investigated.

PG synthesis has been investigated initially as a function of the exposure time. Data show that at least 4 h of exposure to PEMFs is necessary to induce a significant increase in PG synthesis. Greater effects are associated with a longer exposure time, the maximum effect being observed at 24 h. These results confirm previous observations showing that long exposure times, at least 6–9 h, are needed to stimulate proliferation in human primary cells including osteoblast-like cells, lymphocytes and chondrocytes[1,16,35]. It has been reported that 7 h/day exposure to PEMFs stimulates endochondral ossification in rats, by increasing PG synthesis[21]. *In vivo* Fini *et al.*[25] found it was necessary to expose Dunkin Hartley guinea pigs to PEMFs 6 h/day to prevent knee OA progression. Other authors, using different PEMFs demonstrated that 1 h daily exposure could prevent OA progression in Dunkin Hartley guinea pigs[24]. This is relatively surprising considering that besides exposure time, also pulse characteristics may influence the biological response.

The dose–response curve for the effect of magnetic field peak amplitude on PG synthesis shows that all magnetic field values investigated induced a significant increase in PG synthesis. The largest increase was observed at 1.5 mT and then it stabilized, the slight decrease observed at 2 mT being not significant. In different experimental models, other authors observed that maximal effects were reached for magnetic field values of 1–1.5 mT when a range of values of 0.1–2.5 mT was investigated[26,37,38]. The magnetic field peak values that stimulate PG synthesis are of the same amplitude as those used to modulate ligand–receptor kinetics[26,37,38] i.e., 1.5 mT. However, in ligand–receptor studies, the PEMF effect is quite rapid, a few minutes, suggesting that all cell structures, sensitive to the field effects, are immediately and completely activated provided that the field amplitude is above 1 mT. On the contrary, the exposure time required to trigger a significant cellular response is much longer, 4 h in cartilage explants. We interpreted the need for a lengthy PEMF exposure to elicit a biological response as a means of protection from external perturbations of more complex biological systems, such as explants.

Very few studies in the literature investigate the effect of frequency; concerning bone, the PEMF-induced effect was maximal at 15 Hz, and decreased at frequencies under 10 Hz[29,30]. To our knowledge, the influence of field frequency has not been previously investigated in cartilage; we did not find any difference in the PEMF-induced increase in PG synthesis when changing pulse frequency. Also Canè *et al.*[5] did not observe differences by changing frequency in a model of osteogenesis in the horse. It is intriguing why no effect of varying frequency was observed. The time the biological system is actually exposed to the PEMF might depend both on the number of hours of exposure and on the pulse frequency used. In all our experiments, the individual pulse duration was 1.3 ms. This means that during the 24 h of exposure, the induced electric field was present 140 min at 75 Hz and only 3.7 min at 2 Hz. However, we observed that the effect on PG synthesis was not dependent on the frequency used, but on the exposure time only. This emphasizes the importance of the repetition over time of the stimulus to overcome the "biological inertia" of complex systems. In addition, bone requires prolonged stimuli for the functional adaptation to mechanical load. Overall, we should consider that the need for a repetition over time results in a protection of biological systems from environmental physical perturbation. Furthermore, it is noteworthy that PEMF daily exposure, over months, has been used in clinical studies[11,39].

Our results show that PEMFs have a significant effect on cartilage by increasing PG synthesis and promoting anabolic activity in explants, and that this effect can be optimized at least within the range of parameters investigated herein. To what extent the combinations of the most effective exposure conditions may further optimize the response of cartilage explants to PEMFs require further investigations. This is consistent with recent findings indicating that PEMFs have an adenosine-agonist effect[26,37], which has been shown to have anti-inflammatory properties and ultimately provide chondral protection[25,40,41].

The study of the PEMF parameters and exposure conditions to optimize the effects on cartilage is part of the pre-clinical work necessary to identify the most effective treatment regimens for cartilage protection to be tested in clinical trials.

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